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Virus-Specific $CD4^+$ and $CD8^+$ Cytotoxic T-Cell Responses and Long-Term T-Cell Memory in Individuals Vaccinated against Polio

Rahnuma Wahid,[1] Martin J. Cannon,[1] and Marie Chow[1,2]*

Departments of Microbiology and Immunology[1] and Pathology,[2] University of Arkansas for Medical Sciences, Little Rock, Arkansas, 72205

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The presence of poliovirus (PV)-specific $CD4^+$ T cells in individuals vaccinated against polio has been shown, but $CD8^+$ T-cell responses have not been described. Here, we functionally characterize the $CD4^+$ T-cell response and show for the first time that dendritic cells and macrophages can stimulate PV-specific $CD8^+$ T-cell responses in vitro from vaccinees. Both $CD4^+$ T and $CD8^+$ T cells secrete gamma interferon in response to PV antigens and are cytotoxic via the perforin/granzyme B-mediated pathway. Furthermore, the T cells also recognize and kill Sabin 1 vaccine-infected targets. The macrophage-stimulated $CD4^+$ T and $CD8^+$ T cells most likely represent memory T cells that persist for long periods in vaccinated individuals. Thus, immunity to PV vaccination involves not only an effective neutralizing antibody titer but also long-term $CD4^+$ and $CD8^+$ cytotoxic T-cell responses.

Immunity to poliovirus (PV) as a result of a vaccination regimen with oral Sabin vaccine (OPV) and/or inactivated Salk vaccine (IPV) has been the focus of many studies. The majority of these studies have been directed towards the protective neutralizing antibody response in various populations worldwide (6, 10, 15, 28, 45, 49); less attention has been given to adaptive T-cell responses and the probable role of these cells in the resolution of PV infection.

T-cell responses to PV have been studied primarily in mice. These studies have identified several epitopes in PV structural proteins that are recognized by murine CD4 and CD8 T cells (17, 21, 23, 26). As normal mice lack the human poliovirus receptor (PVR) and are not, therefore, susceptible to poliovirus infection, the role of the PV-specific T cells in protective immunity in these mice could not be determined. However, adoptive transfers of PV-specific CD4 T cells into PVR-transgenic mice that are susceptible to PV infection showed that CD4 T cells do indeed provide protection against infection by lethal doses of PV and that these CD4 T cells provide help to B cells to produce protective antibodies (26). In contrast, studies identifying PV-specific T cells present in humans, the normal host for PV, have been limited. It is clear that PV-specific CD4 T cells are induced in vaccinated individuals, and some CD4 T-cell epitopes have been defined (13, 43). There are no previous reports that a CD8 T-cell-mediated response to PV is initiated in vaccinated individuals, even following recent IPV boosters (20), or in primates that are susceptible to PV infection.

The induction of cytotoxic CD8 T cells is a cornerstone of viral immunity, as CD8 T cells are important in viral clearance. Dendritic cells (DCs) and macrophages (Mϕ) are central to the initiation of CD4 and CD8 T-cell responses to pathogens. We have recently shown that these critical antigen-presenting cells (APCs) express PVR and can be productively infected with PV, with kinetics and cytopathology similar to those seen during infection of HeLa cells, and that infection results in apoptotic cell death (48). Several processes that are important in the presentation of antigen and stimulation of T cells are inhibited in infected APCs, including the inhibition of host protein synthesis and receptor-mediated endocytosis. The secretory pathway is inhibited in PV-infected cells and, thus, presentation of antigens in the context of HLA class I molecules on the surface of the infected cells is significantly reduced (5, 7). This suggests that the absence of documented PV-specific CD8 T-cell responses was due to the inability of PV-infected APCs to present endogenous peptides on HLA class I molecules. Thus, we were interested as to whether infected APCs could stimulate CD8 T-cell responses in vitro from individuals who have received OPV.

We show here that PV-specific CD4 and, surprisingly, CD8 T-cell responses are recovered following in vitro stimulation with PV-infected or antigen-loaded DCs. Both CD4 and CD8 T cells secrete gamma interferon (IFN-γ) in response to PV antigens and are cytotoxic via the perforin/granzyme B-mediated pathway. We further show that PV-infected or antigen-loaded Mϕ could also stimulate PV-specific CD4 T-cell and CD8 T-cell responses, suggesting that vaccination with OPV results in the induction of long-lasting PV-specific CD4 and CD8 memory T-cell responses.

MATERIALS AND METHODS

Donors. This study was approved by the Institutional Review Board of the University of Arkansas for Medical Sciences with a minimal-risk-for-adults rating. Six adult volunteers (between the ages of 25 and 65 years) each donated 1 unit of blood for this study. According to the health questionnaire completed by the donors, they had all received OPV, with the exception of one donor who had received both OPV and IPV.

Virus stocks. Poliovirus serotype 1 strains were used for all studies. Mahoney (PVM) and Sabin 1 (PVS) viral stocks were propagated as previously described from a plaque obtained by transfection of the infectious cDNAs into HeLa cells (39).

Antibodies. Monoclonal antibodies (MAbs) directed against human surface markers CD1a, CD14, CD54, CD58, CD68, CD83, CD86, HLA-DR, and HLA

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, 4301 W. Markham St., Slot 511, Little Rock, AR 72205. Phone: (501) 686-5155. Fax: (501) 686-5362. E-mail: chowmarie@uams.edu.

class I (Caltag Laboratories, Burlingame, CA) and against CCR7 and CD45RO (BD Biosciences, San Diego, CA) were used at supplier-recommended concentrations. All were either phycoerythrin- or fluorescein-conjugated antibodies. Antibody-stained cells were analyzed by FACSCalibur (BD Biosciences) using CellQuest Analysis software.

UV-inactivated poliovirus (uvPV). Poliovirus infectivity was inactivated using the UV Cross-linker FB-UVXL-1000 (Fisher Biotech, Pittsburgh, PA). The Mahoney strain was irradiated in serum-free RPMI (10^9 PFU/ml; 35-mm dish) on ice at a distance of 10 cm for 10 min at optimal cross-linking values (1,200 units × 100 μJ/cm^2). These conditions are sufficient to reduce the viral infectivity to below that detectable by plaque assays on confluent HeLa cell monolayers (data not shown). Binding assays showed that UV inactivation did not alter the ability of radiolabeled virus to bind the PVR on HeLa cells (data not shown).

Monocyte isolation and dendritic cell and macrophage culture. Peripheral blood mononuclear cells (PBMCs) from individual donors were isolated from heparinized blood by centrifugation on Ficoll-Hypaque (Bio-One, Inc., Longwood, FL). The buffy coats were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 2 mM glutamine and 10% human AB serum (Valley Biomedical, Inc., Winchester, VA) (RPMI-10). Monocytes from the PBMCs were allowed to adhere to plastic in six-well dishes for 2 h at 37°C and 7% CO_2, and APCs were cultured according to established protocols (34, 38). The nonadherent cells were subsequently used to culture T cells (see below). Monocytes were cultured in fresh RPMI-10 containing 800 U/ml granulocyte-macrophage colony-stimulating factor and 500 U/ml interleukin 4 (IL-4; R&D Systems, Minneapolis, MN) for 5 days to obtain immature DCs. Mature DCs were generated from immature DCs by the addition of 1,000 U/ml tumor necrosis factor alpha (TNF-α; R&D Systems) and 1 mM prostaglandin E_2 (Sigma Chemicals, St. Louis, MO) for 48 h. To generate Mϕ, monocytes were maintained in RPMI-10 containing 100 U/ml macrophage colony-stimulating factor (R&D Systems) for 6 days. All cultures were supplemented with fresh medium and cytokines every 3 days. Monocyte-derived cells were maintained at 37°C and 7% CO_2. The phenotypes of the resultant DC (CD14$^-$, CD83$^+$, HLA-DR$^+$, HLA class I$^+$, CD86$^+$, CD54$^+$, CD58$^+$) and Mϕ (CD14$^+$, CD1a$^+$, and CD68$^+$) populations were confirmed by flow cytometry. The resultant populations were greater than 95% pure.

Poliovirus-infected and uvPV-loaded APCs. Adherent Mϕ were removed from culture plates following the 15-min treatment with 50 mM EDTA-PBS at 37°C and then washed with PBS before they were infected with PV. Mature DCs are nonadherent and were gently aspirated off the culture plates. Infectious PV was bound to APCs on day 7 of culture at a multiplicity of infection of 10 for 30 min at room temperature in serum-free RPMI 1640 supplemented with 2 mM glutamine (C-RPMI). The inoculum was removed by aspiration, and infection was initiated with the addition of medium prewarmed to 37°C. The infected mature DCs (DC-PV) or Mϕ (Mϕ-PV) were used to stimulate T-cell cultures (see below). To generate uvPV-loaded APC cultures, an amount of uvPV equivalent to a multiplicity of infection of 10 was bound to immature DCs (on day 5 of culture) or adherent Mϕ (on day 6 of culture) for 30 min at room temperature in C-RPMI, followed by the addition of prewarmed medium (plus maturation cytokines for the DCs). The uvPV-loaded Mϕ (Mϕ-uvPV) or DCs (DC-uvPV) were cultured further for 24 or 48 h, respectively, prior to their use to stimulate T cells. Mϕ-uvPV were removed from the plates with 50 mM EDTA-PBS treatment prior to their addition into T-cell cultures.

T-cell isolation and culture. Monocyte-depleted peripheral blood lymphocytes were grown in RPMI-10 at 37°C and 7% CO_2, and recombinant human IL-2 (20 units/ml) was added after the first 7 days of culture. For each donor, four autologous T-cell cultures (10^7 peripheral blood lymphocytes plus 5×10^5 to 1×10^5 DC or Mϕ in 10 ml RPMI-10) were initiated, using four different APC cultures (DC-PV, DC-uvPV, Mϕ-PV, or Mϕ-uvPV). After 7 days, the T cells were restimulated (passage 2 [P2]) with the appropriate infected or uvPV-loaded DCs or Mϕ. During P2 and P3, the cultures were maintained in 20 to 30 U/ml IL-2. In all subsequent passages (P3 to P8), the T-cell cultures were passaged every 14 days and the infected or uvPV-loaded APCs were γ irradiated (2,500 rads) prior to their use.

After three passages, the proportion of CD4 and CD8 T cells present was analyzed by flow cytometry, and CD4 and CD8 T cells were purified from each of the four P3 T-cell cultures (T cells that were cultured by stimulating with DC-PV [$T_{DC\text{-}PV}$], DC-uvPV [$T_{DC\text{-}uvPV}$], Mϕ-PV [$T_{M\phi\text{-}PV}$], and Mϕ-uvPV [$T_{M\phi\text{-}uvPV}$]) by positive selection using magnetic beads (Dynal Biotech, Inc., Lake Success, N.Y.) according to the manufacturer's instructions. After purification, the T cells (typically 98% pure as determined by flow cytometry) were resuspended in RPMI-10 and passaged every 14 days with the corresponding γ-irradiated APCs (DC-PV, DC-uvPV, Mϕ-PV, or Mϕ-uvPV). The CD4 T-cell cultures were maintained in 50 U/ml IL-2 and the CD8 T cells in 100 U/ml IL-2, with fresh IL-2 being added every 2 to 3 days.

Proliferation assays. T cells (10^4 cells/well) were incubated for 4 days in quadruplicate wells (200 μl/well in U-bottomed 96-well plates; 37°C, 7% CO_2) in medium alone or in the presence of uninfected, uvPV-loaded, or infected APCs (10^3 cells/well). Blocking MAbs (50 μg/ml, final concentration) specific for HLA class II (L243) or HLA class I (W6/32) were added where indicated at the beginning of the assay to test for HLA class II or class I restriction, respectively. [*methyl*-^{3}H]thymidine (1 μCi/well, 35 Ci/mmol; ICN Biomedical, Inc., Irvine, CA) was added during the last 16 h of the assay. The cells were harvested onto glass fiber filters, and tritiated thymidine incorporation was measured with a Matrix Direct Beta Counter 96 (Packard, Meriden, CT). Proliferation indices (PI) were calculated by the following formula: PI = (cpm_{APC+T} -$cpm_{APC\ alone}$)/$cpm_{T\ alone}$.

Cytokine production. Uninfected, uvPV-loaded, or virus-infected APCs (mature DCs or Mϕ; 10^4 cells/1 ml RPMI-10 in 12-well plates) were incubated overnight with autologous CD4 or CD8 T cells (10^5 cells/well) at 37°C and 7% CO_2. As controls, T cells were incubated alone or with phorbol-12-myristate-13 acetate (PMA; 50 ng/ml) and ionomycin (500 ng/ml). Supernatants (500 μl) were collected, centrifuged (10,000 × *g* for 30 s) to remove particulates, and frozen at −20°C. The supernatants were analyzed for the presence of secreted cytokines using a Human Th1/Th2 Cytokine Cytometric Bead Array system (BD Biosciences), which allows the quantitative detection of multiple cytokines simultaneously from each sample. The samples were analyzed for IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ according to the manufacturer's protocol. Levels of secreted IFN-γ in these supernatants were measured in duplicate samples also using the Quantikine IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's protocols. The assays were read with a Microplate Autoreader (Bio-Tek Instruments, Winooski, VT) at 450 nm.

Cytotoxicity assays. Both the ^{51}Cr release and granzyme B assays were performed using targets that were mock-infected DCs, uvPV-loaded DCs, DCs infected with PVM or PVS for 2 h, or the human NK-sensitive K562 cells. For the ^{51}Cr release assay, the target cells were labeled by incubating with chromium-51 (50 μCi/10^6 cells and 200 μl PBS) (NEN Perkin-Elmer, Boston, MA) for 1 h at 37°C and 7% CO_2, washed, resuspended in RPMI-10, and incubated in triplicate (10^4 target cells/well) with various ratios of T-cell effectors. The assays were harvested after 3 h at 37°C and 7% CO_2 and the supernatants counted. Where indicated, an HLA-specific blocking MAb (L243 or W6/32; 50 μg/ml, final concentration) or concanamycin A (CMA; 16 μM, final concentration) was added at the beginning of the assay. Percent-specific ^{51}Cr release from lysed target cells was calculated as follows: 100 × [cpm (sample release) − cpm (spontaneous release)]/[cpm (total release) − cpm (spontaneous release)]. Spontaneous ^{51}Cr release from uninfected targets in the absence of T cells was <10%. The release of granzyme B by the T cells in response to antigen-loaded or PV-infected targets was also examined, by collecting supernatants from wells containing T cells (10^5 cells/well in quadruplicate) and DCs (10^4/well) incubated as described for the cytotoxicity assays. The levels of granzyme B released during the T-cell and DC interactions were assessed using the Pelikine Compact Human Granzyme B ELISA kit (Research Diagnostics Inc, Flanders, N.J.) according to the manufacturer's protocol.

RESULTS

Previous methods to identify PV-specific T-cell responses in polio vaccinees employed irradiated PBMCs as APCs (43). As the majority of the PBMCs (B and T cells) do not express the poliovirus receptor and so cannot be infected by PV, they were infected with recombinant vaccinia viruses which expressed PV capsid proteins as a source of viral antigen (43). Although CD4 T cells were identified from these cultures, virus-specific CD8 T cells were not reported. However, it has recently been demonstrated that professional APCs, such as DCs and Mϕ, can efficiently stimulate CD4 and also CD8 T cells specific to a variety of antigens (31, 33, 41, 42). This provided an opportunity to characterize further the poliovirus-specific CD4 and, potentially, CD8 T-cell populations that could be induced in polio vaccinees.

DCs differ from Mϕ in that DCs are able to induce both primary and secondary T-cell responses, whereas Mϕ are in-

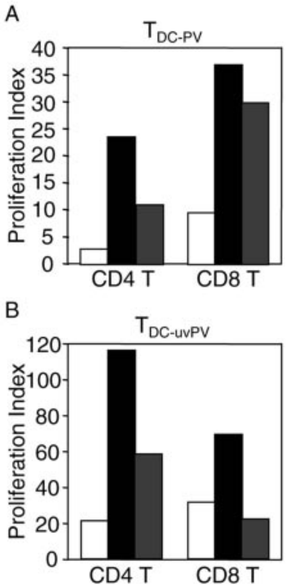

FIG. 1. Dendritic cells stimulate proliferation of PV-specific T cells. CD4 and CD8 T cells were grown in culture by stimulation with DC infected with live PV ($T_{DC\text{-}PV}$) (A) or DC loaded with uvPV ($T_{DC\text{-}uvPV}$) (B). CD4 and CD8 T cells from these cultures were incubated with either DCs alone (white bars) or with DC-uvPV cells (black bars). Cells were also incubated with DC-uvPV in the presence of HLA class II (CD4 T cell)- or HLA class I (CD8 T cell)-blocking antibodies (gray bars). After 72 h, the induced proliferative responses were measured by [^{3}H]thymidine incorporation for 16 h. Representative results from a single donor are shown.

efficient stimulators of naïve T cells but can stimulate secondary recall responses from memory T-cell populations (2, 14). To determine the presence of PV-specific memory T cells in PV-vaccinated individuals, we used infected Mϕ to stimulate T-cell cultures in vitro. Thus, the characterization of the T-cell populations induced by infected DCs and Mϕ should provide a comprehensive study of the PV-specific T-cell-mediated response in vaccinated individuals. A potential limitation of this approach, however, is that both DCs and Mϕ can be productively infected by PV, which inhibits several important cellular functions associated with antigen processing and presentation (48). To allow for this limitation, T-cell cultures were initiated also with APCs loaded with noninfectious uvPV, in parallel to the cultures using APCs infected with the Mahoney strain of PV. The specificity and functions of the resultant T-cell populations were characterized following four to five passages with either PV-infected or uvPV-loaded APCs.

DCs can be used to stimulate PV-specific T cells. In contrast to previous studies and our expectations, PV-specific CD8 T cells as well as CD4 T-cell populations were obtained for all donors from cultures stimulated with either PV-infected DCs (DC-PV) or uvPV-loaded DCs (DC-uvPV). The specificity of these T-cell subsets was initially determined by proliferation assays (Fig. 1). T cells that were cultured by stimulating with DC-PV (Fig. 1A, $T_{DC\text{-}PV}$) or DC-uvPV (Fig. 1B, $T_{DC\text{-}uvPV}$) proliferated specifically in response to DCs loaded with uvPV. PV-specific proliferation of both CD4 and CD8 T-cell subsets could be lowered by the appropriate HLA-blocking antibodies, demonstrating that the T cells proliferated in an HLA-restricted manner.

Although the CD8 T-cell populations proliferated readily in response to uvPV-loaded DCs in these assays, there were noticeable differences in the growth of different CD8 T-cell populations between donors. CD8 $T_{DC\text{-}uvPV}$ cells consistently grew well in culture. In contrast, CD8 $T_{DC\text{-}PV}$ cells did not grow well in culture and could not be grown reproducibly to passages beyond P4/5. This suggested that the T cells were not being efficiently stimulated in vitro by the infected DCs and is consistent with previous data showing that antigen presentation by HLA class I molecules is inhibited in PV-infected cells (5). PV infection of DCs may thus inhibit HLA class I presentation of poliovirus antigens and prevent infected DCs from effectively stimulating the CD8 T cells, thereby explaining the poor growth of the CD8 $T_{DC\text{-}PV}$ cells in vitro. CD4 T-cell populations were obtained upon culture with either infected or uvPV-loaded DCs. This is consistent with previous data indicating that PV infection of DCs does not affect HLA class II presentation of viral antigen and, thus, that infected DCs should be able to effectively stimulate CD4 T cells (48).

Macrophages can stimulate PV-specific memory T cells. As DCs can stimulate both primary and secondary T-cell responses, it was unclear whether the PV-specific cells stimulated by DCs were recall responses from the memory pool or primary responses from naïve T cells. Mϕ are not known to be efficient stimulators of antigen-specific primary T-cell responses (12, 24, 25, 35). Thus, virus-specific T-cell cultures arising from coculture with Mϕ most likely originate from a memory T-cell population in the vaccinated donors. The existence of this memory population is consistent with data from Krieg et al. demonstrating the presence of gut-homing PV-specific T cells in donors boosted with killed vaccine just prior to initiation of that study. The PV-specific T cells characterized in the study were mainly of the effector memory phenotype ($CD4^+CD45RO^+CCR7^-$) (20). PV-specific T cells were induced in cultures stimulated by Mϕ-PV or Mϕ-uvPV. The specificity of the CD4 and CD8 T cells was determined in proliferation assays. CD4 T cells cultured by stimulating with Mϕ-PV (Fig. 2A) or Mϕ-uvPV (Fig. 2B) proliferated specifically in response to Mϕ loaded with uvPV and were thus considered to have been expanded from a pool of PV-specific memory CD4 T cells. Consistent with this interpretation, these cells when analyzed by flow cytometry were positive for expression of CD45RO and negative for CCR7 (data not shown). CD8 T cells from cultures stimulated with Mϕ-PV did not proliferate in response to uvPV-loaded Mϕ (Fig. 2A). Surprisingly, however, CD8 T cells cultured by stimulating with Mϕ-uvPV (Fig. 2B) proliferated specifically in response to uvPV-loaded Mϕ. CD8 T cells responsive to Mϕ-uvPV were found for all donors. The demonstration of the presence of virus-specific CD8 T cells (CD8 T cells that can be expanded in vitro upon stimulation with Mϕ loaded with uvPV) in all donors suggests that vaccinated individuals retain long-term PV-specific CD8 T-cell memory.

The isolation of virus-responsive CD4 and CD8 T-cell populations as a result of DC or Mϕ stimulation provided an opportunity to characterize the phenotypes of these cells. As DCs can stimulate naïve T cells, it is possible that the DC-stimulated cultures may give rise to a population of T cells in

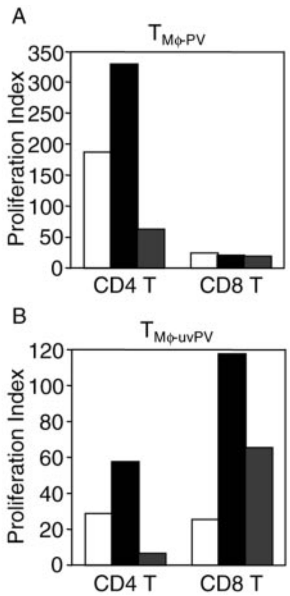

FIG. 2. Macrophages stimulate proliferation of PV-specific memory T cells. CD4 and CD8 T cells were grown in culture by stimulation with Mϕ/($T_{M\phi\text{-}PV}$)/($T_{M\phi\text{-}uvPV}$) infected with live PV (A) or Mϕ loaded with uvPV (B). CD4 and CD8 T cells from these cultures were incubated either with Mϕ alone (white bars) or with Mϕ-uvPV (black bars). T cells were also incubated with Mϕ-uvPV in the presence of HLA class II- or HLA class I-blocking antibodies as appropriate (gray bars). After 72 h, the proliferative response of the cells was measured by the levels of [^{3}H]thymidine incorporated during 16 h. Results from a single donor are represented.

vitro that is significantly different from a memory population of cells stimulated by Mϕ. In examining the phenotype of these T cells, we asked two specific questions. First, were there phenotypic and functional differences between the T cells obtained from the DC and Mϕ (live PV-infected or uvPV-loaded) stimulated cultures? Second, could PV-specific T cells recognize and kill virus infected cells? That is, can the virus-specific CD4 and CD8 T cells play a role in virus clearance and, therefore, in protective immunity? The cells were analyzed in parallel to determine whether the T cells obtained from DC cultures were similar to the PV-specific memory T-cell population obtained from Mϕ cultures and whether the CD4 and CD8 T cells were able to recognize and kill PV-infected cells.

CD4 T cells secrete IFN-γ in the presence of PV antigens. CD4 T cells can be polarized towards the Th1 or Th2 cell phenotype, characterized largely by cytokine expression (29). The PV-specific T cells were examined for their cytokine profile using a Human Th1/Th2 Cytokine Cytometric Bead Array system. This allowed the simultaneous analysis for the presence of IL-2, IL-4, IL-6, IL-10, TNF-α, and/or IFN-γ in the medium of antigen-stimulated T cells. The cytokines produced by each of the CD4 T-cell populations (DC-PV, DC-uvPV, Mϕ-PV, and Mϕ-uvPV) upon stimulation with viral antigens were IL-2, TNF-α, and IFN-γ, all characteristic of the strongly antiviral Th1 phenotype (Table 1). The results shown in Table 1 are the levels of cytokines secreted in response to uvPV-loaded DCs from CD4 $T_{DC\text{-}uvPV}$. T cells alone produced no cytokine and in the presence of DC alone produced negligible levels of cytokine (<30 to 50 pg/ml). Although the levels of each cytokine produced varied by donor, the Th1 phenotype was true of the various CD4 T-cell types from all of the donors.

TABLE 1. Concentrations of cytokines from CD4 $T_{DC\text{-}uvPV}$ cells from CBA analysis[a]

Donor	Cytokines concn (pg/ml)					
	IFN-γ	TNF-α	IL-10	IL-6	IL-4	IL-2
1	1,454	990	5	3	30	104
2	549	128	1	14	19	158
3	1,973	>5,000	116	>5,000	197	4,900
4	1,699	443	0	6	28	3,230

[a] CBA analysis was performed with the T cells of only four of the six donors. The results for all six donors were confirmed by ELISA.

Since virally infected cells in the body can be cleared by T cells that secrete IFN-γ (3, 32, 40, 46), the ability of PV-specific CD4 T cells to produce IFN-γ was examined further. IFN-γ levels in the culture media were measured following coculture of CD4 T cells with APCs either infected with PV or loaded with uvPV. The CD4 $T_{DC\text{-}PV}$ cells (Fig. 3A) and CD4 $T_{M\phi\text{-}PV}$ cells (Fig. 3B) produced IFN-γ when incubated with DC-uvPV. CD4 $T_{DC\text{-}uvPV}$ (Fig. 3C) and CD4 $T_{M\phi\text{-}uvPV}$ (Fig. 3D) behaved in a manner similar to CD4 $T_{DC\text{-}PV}$ and CD4 $T_{M\phi\text{-}PV}$. The responses could be blocked by HLA class II-specific MAbs, demonstrating that this response was dependent on the presentation of antigen in the context of HLA class II molecules.

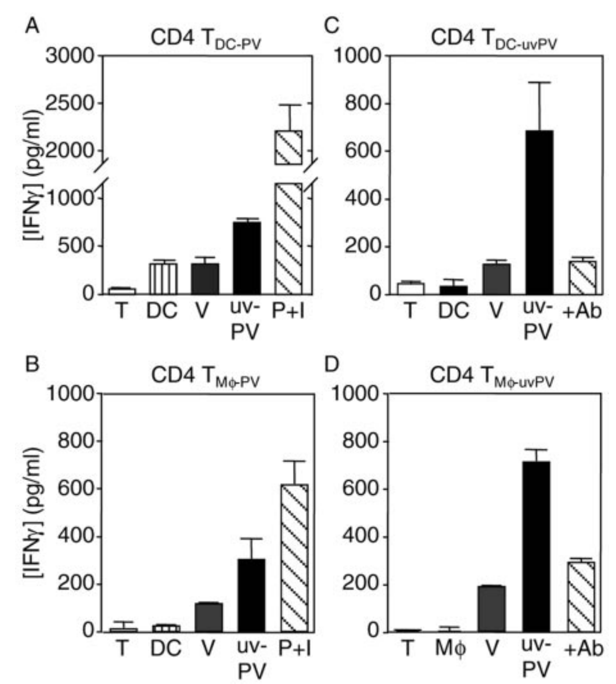

FIG. 3. PV-specific CD4 T cells secrete IFN-γ in the presence of PV antigens. CD4 $T_{DC\text{-}PV}$ (A), CD4 $T_{M\phi\text{-}PV}$ (B), and CD4 $T_{DC\text{-}uvPV}$ (C) cells were incubated overnight with DCs alone (DC), infected DCs (V), or DC-uvPV (uv-PV). CD4 $T_{M\phi\text{-}uvPV}$ (D) cells were incubated overnight with Mϕ alone (Mϕ), infected Mϕ (V), or Mϕ-uvPV (uv-PV). Cells were also stimulated with PMA and ionomycin (P+I) as a positive control or incubated with HLA class II-blocking antibody (+Ab). Supernatants were collected. IFN-γ levels were assayed by ELISA. Representative data for cells of one donor are shown.

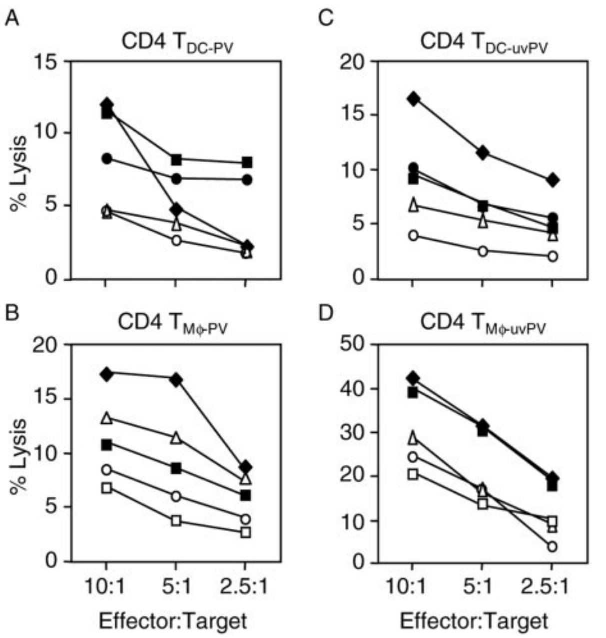

FIG. 4. PV-specific CD4 T cells lyse targets via a perforin-mediated pathway. CD4 $T_{DC\text{-}PV}$ (A), CD4 $T_{M\phi\text{-}PV}$ (B), CD4 $T_{DC\text{-}uvPV}$ (C), and CD4 $T_{M\phi\text{-}uvPV}$ (D) cells were used as effector cells at different ratios in 3-h CTL assays with chromium-loaded targets. Targets were DCs alone (○); DCs infected with PVS (●), PVM (■), or PVM and CMA (□); or DCs loaded with uvPV (◆) or with uvPV and CMA (△). As controls, T cells were also incubated with DC-uvPV in the presence of an HLA class II-blocking antibody (L243) or with K562 cells (not shown). Representative results from the T cells of a single donor are shown.

Thus, irrespective of the APC (DC versus Mϕ) or the source of antigen (infection versus loading of UV-inactivated virus) used to culture the CD4 T cells, these CD4 T cells, when cocultured overnight with uvPV-loaded APCs, produced similar and large amounts of IFN. These results suggest that PV-specific CD4 T cells may play an active role in viral clearance, which is distinct from the established ability of these cells to aid in the production of PV-specific antibodies (26).

CD4 T cells are cytotoxic. In addition to the production of IFN-γ, the PV-specific CD4 T cells were examined for their cytolytic abilities in ^{51}Cr release assays (Fig. 4). The DC-stimulated CD4 T cells (Fig. 4A and C) and the Mϕ-stimulated CD4 T cells (Fig. 4B and D) lysed uvPV-loaded DC targets. Interestingly, CD4 $T_{M\phi\text{-}uvPV}$ from all donors appeared to have higher cytotoxic activity to DC targets loaded with uvPV (Fig. 4D). The reason for this is unclear. Recognition and lysis by all PV-specific responder CD4 T cells could be decreased by the presence of an HLA class II-blocking antibody, L243 (data not shown). Lysis was also determined to be mediated at least in part by the perforin pathway, as the presence of CMA decreased lysis of uvPV-loaded targets (Fig. 4A and C). CMA acts by inhibiting perforin-dependent cytotoxicity through the elevation of granule pH and the accelerated destabilization and degradation of perforin (16).

The Mϕ-uvPV and Mϕ-PV CD4 T cells recognized and lysed infected DC targets (Fig. 4B and D). Although the percentage of lysis was low (Fig. 4A and C), the CD4 T cells cultured using infected or uvPV-loaded DCs showed levels of cytotoxicity similar to those seen with the Mϕ-stimulated CD4 T cells. The CD4 T cells were consistently cytotoxic to DC targets infected with either the PVM or PVS strain (Fig. 4A and C), demonstrating that the T cells not only recognized infected targets but also that PV-specific CD4 T cells were cross-reactive to the vaccine (PVS) and its wild-type parent (PVM) strain of PV. Lysis of infected targets was mediated at least in part via the perforin/granzyme B pathway, as demonstrated by the decreased lysis of infected targets in the presence of CMA (Fig. 4B and D). In separate ELISAs to detect granzyme B, CD4 T cells also released granzyme B upon stimulation with infected or uvPV-loaded APCs (data not shown), further confirming that the perforin/granzyme B-mediated pathway is triggered upon antigen recognition.

Overall, regardless of whether they are possibly de novo stimulated or derived from memory T cells, the CD4 T cells are Th1 polarized, produce IFN-γ in response to PV antigens, and are cytolytic via the perforin/granzyme B pathway. The ability of PV-specific CD4 T cells to recognize PV-infected targets, produce IFN-γ, and subsequently lyse these targets suggests a much broader role for CD4 T cells in immunity to PV than has been hitherto recognized. In addition, PV-specific memory CD4 T cells appear to be long-lasting, as virus-specific T cells can be expanded in vitro by antigen-loaded Mϕ even from individuals who were vaccinated several decades prior to the initiation of this study.

CD8T cells produce IFN-γ. While there is an established role for PV-specific CD4 T cells in protective immunity to PV, the CD8 T cells described in this study represent the first documentation of a PV-specific CD8 T cells response in humans. To examine the potential contributions of the PV-specific CD8 T cells to viral immunity, specific production of IFN-γ and cytotoxic ability was measured. The CD8 $T_{DC\text{-}uvPV}$ cells (Fig. 5C) and CD8 $T_{M\phi\text{-}uvPV}$ (Fig. 5D) secreted significant levels of IFN-γ in response to uvPV-loaded APCs. CD8 $T_{DC\text{-}PV}$ cells (Fig. 5A) produced very low levels of IFN-γ in response to uvPV-loaded DCs; however, IFN-γ production by this stimulated population varied among the donors. Consistent with the failure to generate a PV-specific proliferative response (Fig. 2A), the CD8 $T_{M\phi\text{-}PV}$ cells did not produce significant levels of IFN-γ in response to uvPV (Fig. 5B). CD8 T-cell responses induced with virus-infected APC were thus limited. The CD8 $T_{M\phi\text{-}uvPV}$ cells, that is, the CD8 T-cell population derived from the memory pool using UV-inactivated virus, were the only ones which produced IFN-γ (above levels produced by T cells or DC/Mϕ alone) in response to infected cells. It is not clear why the CD8 $T_{DC\text{-}uvPV}$ cells did not produce IFN-γ in response to infected cells. Nevertheless, these experiments suggest the existence of PV-specific memory CD8 T cells that are responsive to viral antigen (that is, PV-infected or uvPV-loaded DC or Mϕ).

CD8T cells are cytotoxic. HLA class I-restricted CD8 T cells are uniquely able and selected to control virally infected cells by their efficient ability to detect viral peptides derived from intracellular proteins and the subsequent elimination of infected cells by cytolytic mechanisms (51). The potential role of a memory CD8 T-cell population in the vaccinated population in PV-specific immunosurveillance was further tested by examining the cytolytic ability of these cells in vitro using uvPV-

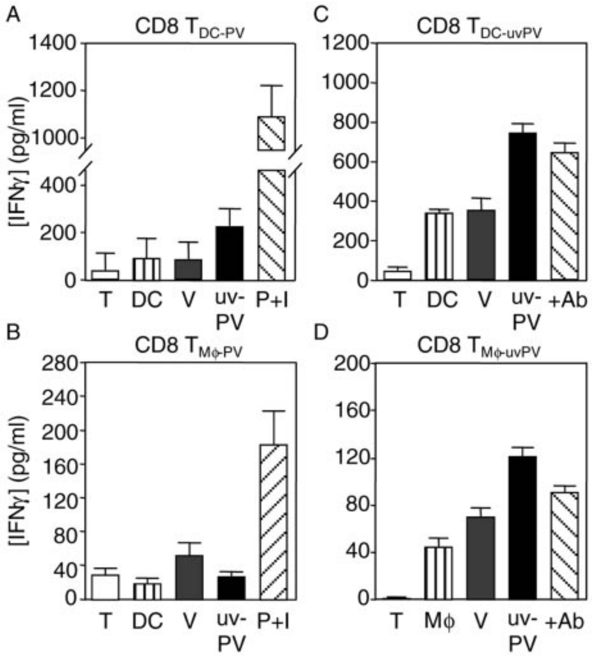

FIG. 5. PV-specific CD8 T cells specifically secrete IFN-γ. CD8 $T_{DC\text{-}PV}$ (A), CD8 $T_{M\phi\text{-}PV}$ (B), and CD8 $T_{DC\text{-}uvPV}$ (C) cells were incubated overnight with DCs alone (DC), infected DCs (V), or DC-uvPV (uv-PV). CD8 $T_{M\phi\text{-}uvPV}$ cells (D) were incubated overnight with Mϕ alone (Mϕ), infected Mϕ (V), or Mϕ-uvPV (uv-PV). T cells incubated with uvPV-loaded APCs were incubated also with HLA class I-blocking antibodies (+Ab) or stimulated with PMA and ionomycin (P+I) where indicated. Supernatants were collected and assayed for levels of IFN-γ by ELISA. Representative data for cells of one donor are shown.

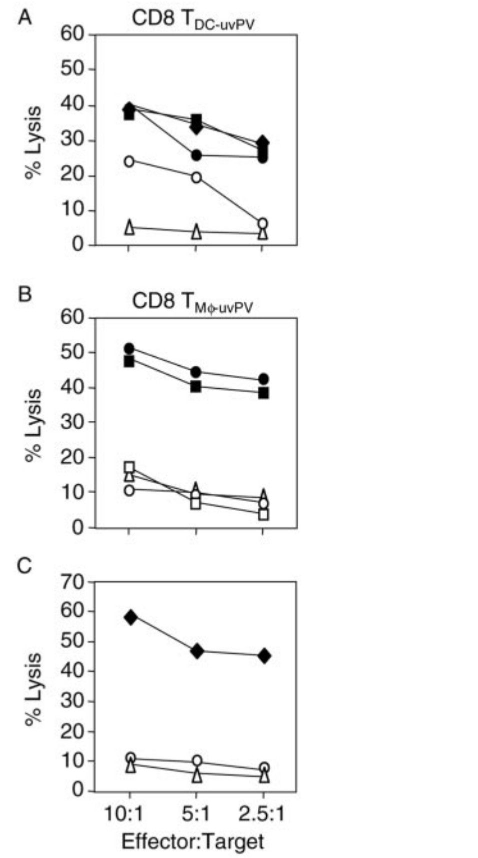

FIG. 6. PV-specific CD8 T cells are cytotoxic. CD8 $T_{DC\text{-}uvPV}$ (A) and CD8 $T_{M\phi\text{-}uvPV}$ (B-C) were incubated at different ratios with chromium-loaded targets in 3-h CTL assays. (A-B) Targets were DCs alone (○), DCs infected with PVS (●) or PVM (■), and DC-uvPV (♦). As controls, T cells were also incubated with PVM-infected DCs in the presence of an HLA class I-blocking antibody (W6/32) (□) and K562 cells (△). (C) CD8 $T_{M\phi\text{-}uvPV}$ cells were incubated with DCs alone (○), with DC-uvPV (♦), and with DC-uvPV in the presence of CMA (△). Representative results from a single donor are shown.

loaded and/or infected targets. As described above, the CD8 $T_{DC\text{-}PV}$ and CD8 $T_{M\phi\text{-}PV}$ cells from all donors did not grow well in culture and, consequently, could not be further characterized for their cytolytic potential.

CD8 $T_{DC\text{-}uvPV}$ and CD8 $T_{M\phi\text{-}uvPV}$ were incubated at different ratios with ^{51}Cr-loaded DC targets in 3-h cytotoxic T-lymphocyte (CTL) assays (Fig. 6) and showed similar specificities and activities. PV-specific CD8 T cells recognized and lysed uvPV-loaded targets (Fig. 6A and C). Especially noteworthy is the fact that the CD8 T cells also lysed infected DC targets (Fig. 6A and B). The CD8 $T_{M\phi\text{-}uvPV}$ recognized and lysed PVS- or PVM-infected targets (Fig. 6B), suggesting that the recall T-cell response was not only cross-reactive to both strains of PV but also that the CD8 T cells present in vaccinees may be important in immunity to the wild-type strain of virus. The NK-sensitive cell line, K562, was not lysed to significant levels by the CD8 T-cell populations (Fig. 6A and B). Furthermore, the T cells were HLA class I restricted, as demonstrated by a decrease in the level of cytotoxicity in the presence of the class I-specific blocking antibody W6/32 (Fig. 6B). The cytotoxicity was mediated mainly by the perforin-dependent pathway (Fig. 6C), as demonstrated by the ability of CMA to abrogate lysis of target DCs. The perforin/granzyme pathway is essential for the control of many viral infections (37). The ability of the PV-specific CD8 T cells to lyse targets via the perforin-mediated pathway is consistent with data from a related picornavirus model, Theiler's murine encephalitis virus (30, 36).

In summary, both DC-stimulated and Mϕ-stimulated CD8 T cells (which appear to be expanded from an existing memory pool in the vaccinated population) produce IFN-γ in response to PV antigens and have cytolytic ability via the perforin/granzyme B pathway. The presence in vaccinated individuals of a PV-specific cytotoxic CD8 T-cell population able to recognize and lyse infected cells (irrespective of the production of IFN-γ), suggests a role for these cells in viral clearance and possible long-term immunity to PV.

DISCUSSION

Durable, long-term immunity to PV is a well-documented feature of oral polio vaccination. These studies demonstrate that virus-specific CD8 as well as CD4 T-cell populations can be recovered from individuals vaccinated with OPV. Both T-cell populations are IFN-γ-producing, cytolytic cells. The phenotype of these cells is relevant to their potential protective roles against PV infection and virus clearance.

Induction of PV-specific CD8 T cells by APCs. CD8 T cells can be induced via several different mechanisms. Although virus-specific CD8 T cells were obtained from all donors, CD8 T cells cultured using PV-infected DCs or Mϕ do not grow well. This suggests that infected APCs may be impaired in their ability to stimulate PV-specific CD8 T cells. Since the stimulation of CD8 T cells requires presentation of antigen in the context of HLA class I molecules on APCs, the impaired growth of CD8 T cells in these cultures is consistent with previous studies demonstrating that HLA class I presentation is significantly reduced in infected cells (5). Of note is that, in contrast to the Mϕ-PV stimulated cultures, PV-infected DCs were capable of inducing at least a limited CD8 T-cell response (Fig. 1A), suggesting that DCs, although highly susceptible to lytic infection by PV (48), retain at least some class I antigen-presenting capability. Thus, it is possible that this capability of infected DCs is important in the generation of PV-specific CD8 T cells in vivo.

In addition, uninfected DCs can acquire viral antigens by endocytosis of nonreplicating antigens (such as uvPV) or apoptotic vesicles (from infected cells), which are subsequently cross-presented on class I molecules (19). Both receptor endocytosed (19, 44) and phagocytically endocytosed antigens can be presented on HLA class I molecules on the surface of DCs (9, 22). Although cross presentation in Mϕ is not as well documented (33, 44), we are able to stimulate CD8 T-cell responses from both DCs and Mϕ loaded with uvPV, indicating the existence of this pathway in Mϕ. It should be noted that we have not determined whether the cross-presentation of uvPV by APCs occurs via binding to PVR, via the Fc receptors (as there is a minimal level of anti-PV antibody present in the human serum used in the T-cell cultures), or via simple phagocytosis. Also, it is not known how the PVR is recycled in the APCs. However, for cross-presentation to occur, PVR complexed with uvPV may enter a pathway similar to that of the Fc receptors, which are known to facilitate cross presentation of captured antigens (1, 8). Cross-presentation of endocytosed polioviral antigens onto HLA class I molecules could result in the induction of virus specific CD8 T cells.

Role of T cells in immunity to PV. The presence of PV-specific CD4 T cells in vaccinated individuals is well documented (4, 43, 47), and virus-specific CD4 T cells are known to provide help to B cells for the production of serotype-specific PV antibodies. Our studies have suggested a more complex role for CD4 T cells in immunity to PV. CD4 T-cell responses are initiated via the cell surface presentation of endocytosed, processed antigens in the context of HLA class II molecules on the cell surface of APCs. In our experiments, PV and uvPV bind to PVR and are subsequently endocytosed and presented on HLA class II molecules. CD4 T cells are efficiently induced by both infected and uvPV-loaded DCs and Mϕ (Fig. 1 and 2). The fact that infected APCs are able to induce PV-specific CD4 T cells supports previous data that HLA class II presentation remains relatively intact in infected APCs (48). Therefore, it is likely that infection of APCs following administration of the OPV results in effective induction of CD4 T-cell responses. The resultant CD4 T cells are not only important in interacting with B cells to stimulate antibody production but also are able to produce IFN-γ and lyse infected target cells. IFN-γ production from these T cells is consistent with previous findings, which showed that recently boosted adults have lymphocytes that proliferate specifically to Sabin antigens and produce large amounts of IFN-γ (20, 47). This cytotoxic capability has been observed also for CD4 T-cell responses to other viruses (11, 18, 50). The cytolytic ability combined with its production of IFN-γ indicates that poliovirus-specific CD4 T cells can play an active role in virus clearance.

An equally important facet of viral immunity is the presence of a CD8 T-cell response. We have shown that PV-infected or antigen-loaded Mϕ stimulate PV-specific cytotoxic CD8 T-cell responses. We further show that the CD8 T cells, when stimulated with uvPV-loaded DCs or Mϕ, are able to produce IFN-γ in the presence of PV antigens and, importantly, are also cytolytic against infected cells. The phenotype of the CD8 T-cell populations strongly supports a role for these cells, also, in the clearance of virus-infected cells. The demonstration of a CD8 T-cell response also revises the common view that virus clearance in PV-infected individuals is solely via the CD4 T-cell/antibody pathways.

Consistent with the view that polio immunity is long lasting, we were able to recover T cells from all of our donors, who were vaccinated over two decades before our studies were conducted. In addition, the ability to obtain PV-specific T cells from uvPV-loaded Mϕ suggests that these T cells are expanded in vitro from low numbers of memory T cells present in vivo (in OPV-vaccinated individuals) and do not represent primary stimulated T cells from a naïve population. This interpretation is strongly supported by previous comparative studies addressing the induction of antiviral proliferative and cytotoxic responses in vitro by DCs and Mϕ (12, 24, 25). A possible caveat to the interpretation that these T-cell responses result from OPV administration is that other enterovirus infections may induce T-cell populations that cross-recognize PV epitope sequences (27). However, because the donors did not report previous histories of enterovirus disease and polio vaccination was the only common factor, we favor the interpretation that these T-cell populations result from polio vaccination. Collectively, these results suggest that following PV vaccination, there is long-term persistence of not only PV-specific memory CD4 T cells but also PV-specific memory CD8 T cells and that the immune mechanisms associated with OPV administration involve both antibody production and cytotoxic CD4 and CD8 T-cell responses.

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